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**DESCRIPTION**

Probes for Detecting Protein Nuclear Transport and Method for Detecting  
and Quantifying Protein Nuclear Transport using the Same

5

**Technical Field**

The invention of this application relates to a pair of probes for  
detecting and quantifying protein nuclear transport induced by action of  
10 bioactive substances. More specifically, the invention of this application  
relates to a pair of probes for detecting and quantifying protein nuclear  
transport induced by action of bioactive substances with high accuracy, a  
method for detecting and quantifying protein nuclear transport using the  
same, and a method for screening substances which induce or inhibit  
15 protein nuclear transport.

**Background Art**

20 The control of complicated signaling networks within eukaryotic  
cells relies on the compartmentalization of each protein. Nucleocytosolic  
trafficking of proteins in response to extra- or intracellular stimuli is an  
essential step for regulating the magnitude and specificity of gene  
expressions. The trafficking is regulated by posttranslational  
25 modifications of proteins, which include ligand-receptor binding, protein  
phosphorylation, and proteolysis.

Because various nuclear proteins are mislocalized in cancer cells,  
there is an intense interest in identifying small molecules that redirect the  
30 proteins to the correct compartments. Also, the nuclear localization of the

proteins is altered in the cells that are exposed to specific exogenous chemicals, of which potential effects on living animals are the major concern.

5 Hence, development of a rapid screening system to detect the nucleocytoplasmic trafficking is essential for the discovery of novel compounds that have anticancer activity or for testing the toxicity of chemicals, from which new insights into the mechanism of nucleocytoplasmic trafficking could be provided (non-Parent Documents 1-4).

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A technique for monitoring the dynamics of the protein movement inside single living cells relies on the use of immunocytochemistry or optical, imaging with genetically tagged GFP (non-Patent Document 5). These analyses are effective for imaging the spatial and temporal dynamics of proteins of interest within single living cells. However, there have been problems that it takes a long time to confirm and quantify the intracellular localization under a microscope, and analyses of the protein localization in living animals require complex assay procedures such as extraction of an organ and dividing it into sliced sections.

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non-Patent Document 1: Kau, T. R. & Silver, P. A. Drug Discov. Today 8,  
78-85 (2003).

non-Patent Document 2: Kau, T. R. et al. Nat. Rev. Cancer 4, 1-12 (2004).

non-Patent Document 3: Rudin, M. & Weissleder, R. Nat. Rev. Drug Discov.  
2, 123-131 (2003).

25

non-Patent Document 4: Gray, L. E., Jr. et al. Toxicology 181-182, 371-382  
(2002).

non-Patent Document 5: Elion, E. A. Methods Enzymol. 351, 607-622  
(2002).

30 non-Patent Document 6: Singh, S. M. et al. Curr. Med. Chem. 7, 211-247

(2000).

non-Patent Document 7: Lorenz, W. W. et al. Proc. Natl. Acad. Sci. USA 88,  
4438-4442 (1991).

5 non-Patent Document 8: Mathews, J. C. et al. Biochemistry 16, 85-91  
(1977).

non-Patent Document 9: Paulmurugan, R. & Gambhir, S. S. Anal. Chem.  
75, 1584-1589 (2003).

non-Patent Document 10: Kaihara, A. et al. Anal. Chem. 75, 4176-4181  
(2003).

10 non-Patent Document 11: Bhaumik, S. & Gambhir, S. S. Proc. Natl. Acad.  
Sci. USA 99, 377-382 (2002).

non-Patent Document 12: Sun, L. et al. Appl. Environ. Microbiol. 67,  
1025-1029 (2001).

15 non-Patent Document 13: Wu, H. et al. Proc. Natl. Acad. Sci. USA 95,  
9226-9231 (1998).

non-Patent Document 14: Yang, J. et al. Proc. Natl. Acad. Sci. USA 100,  
3513-3518 (2003).

non-Patent Document 15: Giriat, I. & Muir, T. W. J. Am. Chem. Soc. 125,  
7180-7181 (2003).

20 non-Patent Document 16: Chin, H. G. et al. Proc. Natl. Acad. Sci. USA 100,  
4510-4515 (2003).

non-Patent Document 17: Ozawa, T. et al. Anal. Chem. 73, 5866-5874  
(2001).

25 non-Patent Document 18: Ozawa, T. et al. Anal. Chem. 73, 2516-2521  
(2001).

non-Patent Document 19: Ozawa, T. et al. Nat. Biotechnol. 21, 287-293  
(2003).

non-Patent Document 20: Paulmurugan, R. et al. Proc. Natl. Acad. Sci.  
USA 99, 15608-15613 (2002).

30 non-Patent Document 21: Massoud, T. F. & Gambhir, S. S. Genes Dev. 17,

545-580 (2003).

non-Patent Document 22: Greer, L. F., 3rd & Szalay, A. A. Luminescence  
17, 43-74 (2002).

5 non-Patent Document 23: Weissleder, R. & Ntziachrostos, V. Nat. Med. 9,  
123-128 (2003).

### Disclosure of the Invention

10 Under the foregoing circumstances, the invention of this  
application has been made, and it aims to provide, upon solving the  
problems associated with the ordinary techniques, a convenient and highly  
accurate method for detecting protein nuclear transport induced by  
endogenous and exogenous substances in local areas of living cells or  
15 living animals.

The invention of this application first provides, for solving the  
foregoing problems, a pair of probes for detecting and quantifying protein  
nuclear transport induced by action of a bioactive substance, comprising

20 Probe I in which a protein whose nuclear transport is to be detected  
or quantified is connected to an N-terminal end or a C-terminal end of a  
fusion protein [intein-C/reporter protein-C] wherein at least a C-terminal  
side polypeptide of an intein and a C-terminal side polypeptide of a  
reporter protein are connected in this order, and

25 Probe II in which a nuclear localization signal is connected to an  
N-terminal end or a C-terminal end of a fusion protein [reporter  
protein-N/intein-N] wherein at least the remaining N-terminal side  
polypeptide of the reporter protein and the remaining N-terminal side  
polypeptide of the intein are connected in this order. The invention of this  
30 application second provides a pair of probes for detecting and quantifying

protein nuclear transport induced by action of a bioactive substance,  
comprising

Probe I in which a protein whose nuclear transport is to be detected  
or quantified is connected to an N-terminal end or a C-terminal end of a  
5 fusion protein [reporter protein-N/intein-N] wherein at least a N-terminal  
side polypeptide of a reporter protein and a N-terminal side polypeptide of  
an intein are connected in this order, and

Probe II in which a nuclear localization signal is connected to an  
N-terminal end or a C-terminal end of a fusion protein [intein-C/reporter  
10 protein-C] wherein at least the remaining C-terminal side polypeptide of  
the intein and the remaining C-terminal side polypeptide of the reporter  
protein are connected in this order.

The invention of this application third provides the pair of probes,  
15 wherein the intein is a DnaE intein derived from blue-green algae, and  
fourth provides the pair of probes, wherein the reporter protein is  
luciferase.

The invention of this application fifth provides a method for  
20 detecting and quantifying protein nuclear transport induced by action of a  
bioactive substance, which comprises

making Probe I of any of foregoing pair of probes and the bioactive  
substance coexist in the cytosol,  
localizing Probe II in the nucleus, and  
25 measuring a signal of the reporter protein within the nucleus.

The invention of this application sixth provides the method for  
detecting and quantifying protein nuclear transport, wherein  
polynucleotides expressing any of foregoing pair of probes are introduced  
30 into a cell thereby making Probe I and the bioactive substance coexist in

the cytosol and localizing Probe II in the nucleus, and seventh provides the method for detecting and quantifying protein nuclear transport, wherein polynucleotides expressing any of the foregoing pairs of probes are introduced into a non-human animal multipotent cell and the cell is  
5 subjected to ontogenesis thereby making Probe I and the bioactive substance coexist in the cytosol and localizing Probe II in the nucleus in all cells of the animal or its progeny.

The invention of this application eighth provides a method for  
10 screening a protein nuclear transport-inducing substance, which comprises

introducing Probe I of any of the foregoing pair of probes into the cytosol,

localizing Probe II in the nucleus,

15 introducing a nuclear transport-inducing candidate substance into the cytosol, and

measuring a signal of the reporter protein in the nucleus.

The invention of this application ninth provides a method for  
20 screening a protein nuclear transport-inhibiting substance, which comprises:

introducing Probe I of any of the foregoing pair of probes into the cytosol,

localizing Probe II in the nucleus,

25 introducing a nuclear transport-inhibiting candidate substance into the cytosol,

further introducing a nuclear transport-inducing substance into the cytosol,

measuring a signal of the reporter protein in the nucleus, and

30 comparing the signal with a signal of the reporter protein obtained

by introducing only the protein nuclear transport-inducing substance into the cytosol.

The invention of this application tenth provides the screening method, wherein polynucleotides expressing any of the foregoing pair of probes are introduced into the cell thereby introducing Probe I into the cytosol and localizing Probe II in the nucleus, and eleventh provides the screening method, wherein polynucleotides expressing any of the foregoing pair of probes are introduced into a non-human animal multipotent cell and the cell is subjected to ontogenesis thereby introducing Probe I in the cytosol and localizing Probe II in the nucleus in all cells of the animal or its progeny.

#### **Brief Description of the Drawings**

Fig. 1 is a schematic view showing a structure and an action of the pair of probes for detecting protein nuclear transport in this invention.

Fig. 2 is a fluorescence microscope image and a transmission microscope image of COS-7 cells transiently transfected with pcDRc-AR expression vector. (a: anti-AR antibody; b: anti-Flag antibody; 1: without addition of DHT; 2: addition of DHT)

Fig. 3 is Western blot in Examples of this invention. (a: protein extract of COS-7 cells; b: cells with probes I and II co-expressed (addition of DHT); c: cells with probes I and II co-expressed (without addition of DHT))

Fig. 4 is dose-response curves of DHT added to cells based on the



luminescence intensities of reconstituted RLuc.

Fig. 5 is dose-response curves of various endogenous hormones and synthetic chemicals based on the luminescence intensities of reconstituted RLuc. (a: endogenous hormones; b: synthetic chemicals; c: inhibitors of protein nuclear transport)

Fig. 6 is a CCD image showing protein nuclear transport in mouse carrying transiently transfected COS-7 cells. (1: no probe; 2: only Probe II is expressed; 3: only Probe I is expressed; 4: probes I and II are expressed)

Fig. 7 is the average of photon counts from mouse carrying transiently transfected COS-7 cells. (1: no probe; 2: only Probe II is expressed; 3: only Probe I is expressed; 4: probes I and II are expressed)

Fig. 8 is a time course of luminescence change after i.p. injection of coelenterazine in mice carrying transiently transfected COS-7 cells. (1: no probe; 2: only Probe II is expressed; 3: only Probe I is expressed; 4: probes I and II are expressed)

Fig. 9 is a CCD image of mice carrying COS-7 cells cotransfected with probes I and II in the presence or absence of DHT.

Fig. 10 is photon counts from mice carrying COS-7 cells cotransfected with probes I and II in the presence or absence of DHT. (average of four mice)

Fig. 11 is a CCD image showing inhibition of protein nuclear transport with procymidone and PCB in mice carrying COS-7 cells cotransfected with probes I and II. (a: control (DMSO stimulus); b: DHT; c: DHT+ procymidone; d: DHT+PCB)



Fig. 12 is a graph showing inhibition of protein nuclear transport with procymidone and PCB in mice carrying COS-7 cells cotransfected with probes I and II. (a: control (DMSO stimulus); b: DHT; C: DHT+  
5 procymidone; d: DHT+PCB)

Fig. 13 is a schematic view showing a structure and an action of the pair of probes for detecting protein nuclear transport in this invention (glucocorticoid receptor as a protein).  
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Fig. 14 is a fluorescence microscope image and transmission microscope image of NIH3T3 cells transiently transfected with pcDRc-GR expression vector (a: anti-GR antibody; b: anti-Flag antibody; Corti-: no addition of corticosterone; Corti+: addition of corticosterone)  
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Fig. 15 is dose-response curves of corticosterone, dexamethasone, cortisol, progesterone, testosterone, 2DG, DHT, Mf(RU486), E2 and C-terminal only (corti) added to cells based on the fluorescence intensities of reconstituted RLuc.  
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Fig. 16 is a CCD image showing a protein nuclear transport in mouse carrying transiently transfected NIH3T3 cells. (1: no probe; 2: only Probe II is expressed; only Probe I is expressed; 4: probes I and II are expressed)  
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Fig. 17 is a schematic view showing a structure and an action of the pair of probes for detecting a protein nuclear transport in this invention (Sterol Regulatory Element-Binding Protein-2 as a protein).

Fig. 18 is a microscope image and a transmission microscope image  
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of cells transiently transfected with pcDRc-SREBP expression vector. (a: detection of N-terminal protein; b: detection of SREBP-2; arrow 1: condition before translocation into the nucleus (localization); arrows 2 to 4: conditions after translocation into the nucleus; +chol: in the presence of cholesterol; -chol: in the absence of cholesterol)

Fig. 19 is dose-response curves of cholesterol added to cells based on the luminescence intensities of reconstituted RLuc.

Fig. 20 is a schematic view showing a structure and an action of the pair of probes for detecting protein nuclear transport in this invention (Signal Transducer and Activator of Transcription 3 as a protein).

Fig. 21 is a fluorescence microscope image and a transmission microscope image of HEK293 cells transiently transfected with pcDRc-STAT-3 expression vector. (a: anti-Flag antibody; b: anti-STAT antibody; OSM-: no addition of OSM; OSM+: addition of OSM)

Fig. 22 is dose-response curves of OSM added to cells based on the fluorescence intensities of reconstituted RLuc.

Incidentally, the reference numerals in the drawings indicate the followings.

- 1: pair of probes for detecting protein nuclear transport
- 11: Probe I
- 12: Probe II
- 2: protein
- 3: reporter protein
- 3c: C-terminal side polypeptide of reporter protein
- 3n: N-terminal side polypeptide of reporter protein

4: intein

4c: C-terminal side polypeptide of intein

4n: N-terminal side polypeptide of intein

5: nuclear localization signal

5 6: bioactive substance, protein nuclear transport-inducing substance

6': protein nuclear transport-inducing candidate substance

6'': protein nuclear transport-inhibiting candidate substance

10 7: linker

8: nuclear membrane

### Best Mode for Carrying Out the Invention

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Fig. 1 has shown a schematic view indicating a principle of the pair of probes for detecting protein nuclear transport in this invention. That is, the pair of probes 1 comprises Probe I (11) and Probe II (12). In Probe I (11), target protein 2 is connected to an N-terminal end or a C-terminal end of a fusion protein [intein-C/reporter protein-C] in which at least a C-terminal side polypeptide 4c of intein 4 and a C-terminal side polypeptide 3c of reporter protein 3 are connected in this order. In Probe II (12), nuclear localization signal 5 is connected to an N-terminal end or a C-terminal end of fusion protein [reporter protein-N/intein-N] in which at least the remaining N-terminal side polypeptide 3n of reporter protein 3 and the remaining N-terminal side polypeptide 4n of intein 4 are connected in this order. Alternatively, in Probe I (11), target protein 2 is connected to an N-terminal end or a C-terminal end of fusion protein [reporter protein-N/intein-N] in which at least an N-terminal side polypeptide 3n of reporter protein 3 and an N-terminal side polypeptide 4n of intein 4 are

connected in this order, and in Probe II (12), nuclear localization signal 5 is connected to an N-terminal end or a C-terminal end of fusion protein [intein-C/reporter protein-C] in which at least the remaining C-terminal side polypeptide 4c of intein 4 and the remaining C-terminal side polypeptide 3c of reporter protein 3 are connected in this order.

When introduced the pair of probes into the cell, Probe I (11) is localized in the cytosol, and Probe II (12) in the nucleus. When the protein 2 in Probe I (11) is bound to nuclear transport-inducing substance 6, Probe I (11) translocates from cytosol C into nucleus N and approaches Probe II (12). Then, two sites 4c, 4n of intein 4 approach and are correctly folded, whereby splicing takes place, intein 4 is cut out from Probe I (11) and Probe II (12), and two sites 3c, 3n of reporter protein 3 are connected via a peptide linkage to reconstitute reporter protein 3.

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Meanwhile, when protein 2 in Probe I (11) is not bound to nuclear transport-inducing substance 6, the splicing of intein 4 does not take place, and reporter protein 3 is not reconstituted accordingly.

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Therefore, the translocation of protein 2 into the nucleus can be detected by measuring the signal of reporter protein 3.

In the foregoing pair of probes, Probe I (11) is a tandem fusion protein having a structure of [protein 2/intein-C 4c/reporter protein-C 3c], [intein-C (4c)/reporter protein-C (3c)/protein (2)], [protein 2/reporter protein-N 3n/intein-N 4n] or [reporter protein-N 3n/intein-N 4n/protein 2]. Such Probe I may contain, other than these elements, a polypeptide or the like as linker sequence 7 between the respective elements.

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Likewise, Probe II (12) is a tandem fusion protein having a

structure of [reporter protein 3n/intein-N 4n/nuclear localization signal 5],  
[nuclear localization signal 5/reporter protein-N 3n/intein-N 4n], [intein-C  
4c/reporter protein-C 3c/nuclear localization signal 5] or [nuclear  
localization signal 5/intein-C 4c/reporter protein-C 3c]. Probe II may  
5 contain, other than these elements, a polypeptide or the like as a linker  
sequence 7 between the respective elements.

In the pair of probes 1, protein 2 contained in Probe I (11) is not  
particularly limited so long as its translocation into the nucleus is to be  
10 detected and quantified. For example, androgen receptor (AR), a  
well-known nuclear hormone receptor is employable. AR is known to  
translocate from the cytosol into the nucleus by being bound to  
5 $\alpha$ -dihydrotestosterone (DHT) (non-Patent Document 6). Further, allyl  
hydrocarbon receptor (AhR) known to translocate into the nucleus by a  
15 dioxin, thyroid hormone receptor (ThR) known to translocate into the  
nucleus by thyroid hormone, sterol responsive element binding protein  
(SREBP) known as a cholesterol sensor, mitogen-activated protein kinase  
(MAPK) involved in a cell growth signal, nuclear factor- $\kappa$ B (NF $\kappa$ B) inducing  
apoptosis and the like are employable.

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In the pair of probes 1, nuclear localization signal 5 contained in  
Probe II (12) is for localizing Probe II (12) in the nucleus *N* when Probe II  
(12) is introduced into cells, and its structure and sequence are not  
particularly limited. Specifically, SEQ ID NO: 1 is employable. Of course,  
25 other than this, known nuclear localization sequences (NLS) such as  
nucleoplasmin-derived sequence (SEQ ID NO: 2) and HIV-1 Rev-derived  
sequence (SEQ ID NO: 3) can be employed.

In the pair of probes 1, reporter protein 3, which is divided into  
30 C-terminal side polypeptide 3c and N-terminal side polypeptide 3n for

Probe I (11) and Probe II (12), may be any reporter protein so long as peptide binding directly takes place by splicing with intein 4 to allow reconstitution of split reporter protein, thereby enabling analysis. For example, a fluorescent protein or a luminous catalytic enzyme is preferably employed. A photoprotein such as a green fluorescent protein (GFP) and its derivatives is preferable because it emits light and allows visual analysis. The luminous catalytic enzyme such as luciferase is also preferable because it forms an active center by being connected and emits light easily detectable with a luminometer. Renilla luciferase (RLuc) is especially preferable because its molecular weight is as low as 36-kDa and ATP or posttranslational modification is not necessary for its activity (non-Patent Documents 7 and 8). In order that the N- and C-terminals of split RLuc respectively do not exhibit fluorescence alone but recover its activity by being bound, it is advisable to split at the RLuc active center. Specifically, it is reported that the active center is preferably dissected between G229 and K230 (non-Patent Documents 9 and 10). Further, RLuc takes an advantage of its substrate, jejunal luciferin (coelenterazine), which rapidly penetrates through cell membranes and produces luminescence intensity sufficient for in vivo visualization (non-Patent Document 11).

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Moreover, in the pair of probes 1, known inteins derived from various organisms can be employed as C-terminal side polypeptide 4c and N-terminal side polypeptide 4n for Probes I (11) and Probe II (12). Intein 4 is preferably a site-specific endonuclease so as to be automatically cut out when protein 2 of Probe I (11) interacts with nuclear transport-inducing substance 6 and Probe I (11) translocates into the nucleus (N) to approach Probe II (12). Specifically, yeast VMA-derived intein and blue-green algae-derived DnaE intein are preferable. Of these, blue-green algae-derived DnaE is easy to handle because the DNA sequence of PCC6803 strain is known, as well as it is natural split intein (non-Patent

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Documents 12 to 20).

In the pair of probes 1, for the splicing of intein 4 to effectively occur, it is required that when Probe I (11) and Probe II (12) approach in the nucleus, the two sites involved in the protein splicing are correctly folded and accurately arranged. Therefore, while any intein derived from organisms may directly be used, it may be preferable to design the intein for easy splicing by converting or deleting some amino acid residues or introducing an appropriate linker sequence.

In the pair of probes 1, protein 2 is connected to the N-terminal end or the C-terminal end of fusion protein [intein-C 4c/reporter protein-C 3c] in which a split polypeptide fragment of intein 4 (for example, C-terminal side: 4c) is connected to a split polypeptide fragment of reporter protein 3 (in this case, 3c) to construct Probe I (11).

Nuclear localization signal 5 is connected to the N-terminal end or the C-terminal end of fusion protein [reporter protein-N 3n/intein-N 4n] in which another split polypeptide fragment of reporter protein 3 (3n in the foregoing example) is connected to another split polypeptide fragment of intein 4 (in this case, N-terminal side: 4n) in this order to construct the Probe II (12).

In such a pair of probes 1, connecting manner of intein-C 4c and reporter protein-C 3c; fusion protein [intein-C (4c)/reporter protein-C (3c)] and protein (2); reporter protein-N 3n and intein-N 4n; the fusion protein [reporter protein-N 3n/intein-N 4n] and nuclear localization signal 5 may be arbitrary unless influencing protein 2, nuclear localization signal 5, Probes (11), (12) and the like. For example, chemical, biochemical or gene engineering method can be applied.



In the invention of this application, a method for detecting and quantifying the translocation of protein 2 into the nucleus using pair of probes 1 is provided. Specifically, Probe I (11) in the pair of probes 1 is caused to coexist with bioactive substance 6 in the cytosol, and Probe II (12) is localized in the nucleus (N) thereby measuring the signal of reporter protein 3 in the nucleus (N).

In this method for detecting and quantifying protein nuclear transport, Probe I (11) is retained in the cytosol (C), and Probe II (12) is localized in the nucleus (N) by nuclear localization signal 5. Protein 2 recognizes and binds to bioactive substance 6, whereby Probe I (11) translocates from the cytosol (C) into the nucleus. Then, Probe I (11) and Probe II (12) approach, and intein 4 therein is cut out by splicing to reconstitute reporter protein 3. Accordingly, when the signal of reporter protein 3 is measured, the translocation of protein 2 into the nucleus by bioactive substance 6 can be detected with high accuracy. A calibration curve of a relation between the concentrations and the signal intensities of reporter protein 3 allows quantifying the degree of the translocation of protein 2 into the nucleus.

In the method of this invention, for coexisting Probe I (11) and bioactive substance 6 in the cytosol (C) and localizing Probe II (12) in the nucleus (N), polynucleotides expressing the pair of probes 1 may be introduced into cells. Alternatively, it is also possible that the polynucleotides expressing the pair of probes 1 are introduced into non-human animal multipotent cells and the cells are subjected to ontogenesis, whereby in all cells of this animal or its progeny, Probe I (11) and bioactive substance 6 are caused to coexist and Probe II (12) is localized in the nucleus (N).

The invention of this application also provides a method for screening a substance that induces translocation of protein 2 into the nucleus. That is, Probe I (11) of the pairs of probes 1 is introduced into the cytosol (C), Probe II (12) is localized in the nucleus (N), and nuclear transport-inducing candidate substance 6' is made to coexist in the cytosol (C). Subsequently, the signal of reporter protein 3 in the nucleus (N) is measured, whereby the nuclear transport-inducing substance can be screened.

When nuclear transport-inducing candidate substance 6' interacts with protein 2 in Probe I (11) to induce the nuclear translocation of protein 2, Probe I (11) translocates from the cytosol (C) into the nucleus (N) and approaches Probe II (12) localized in the nucleus (N). Consequently, a half polypeptide of intein 4c and another half polypeptide of intein 4n approach to cause the splicing, and divided reporter proteins 3c and 3n are peptide-bonded to reconstitute reporter protein 3. Accordingly, by measuring the signal of reporter protein 3, it can be confirmed whether or not the candidate substance is a nuclear transport -inducing substance.

For introducing Probe I (11) into the cytosol (C) and localizing Probe II (12) in the nucleus (N) in this screening method, a method for introducing the polynucleotides expressing pair of probes 1 into cell may be applied. Alternatively, by introducing the polynucleotides expressing pair of probes 1 into a non-human animal multipotent cell and the cell is subjected to ontogenesis, Probe I (11) is introduced into the cytosol and Probe II (12) is localized in the nucleus (N) in all cells of this animal or its progeny.

The invention of this application further provides a method for

screening a protein nuclear transport-inhibiting substance. That is, Probe I (11) of pairs of probes 1 is introduced into the cytosol (C), Probe II (12) is localized in the nucleus (N), and nuclear transport-inhibiting candidate substance 6'' is first introduced into the cytosol (C). Protein nuclear transport-inducing substance 6 is then introduced into the cytosol and the signal of reporter protein 3 in the nucleus (N) is measured. The result thereof is compared with the signal of reporter protein 3 at introduction of protein nuclear transport-inducing substance 6 alone into the cytosol (C), whereby the nuclear transport-inhibiting substance can be screened.

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When the nuclear transport-inhibiting candidate substance 6'' inhibits the binding of protein 2 in Probe I (11) and protein nuclear transport-inducing substance 6, the signal intensity of reporter protein 3 is decreased in comparison to that at the absence of the candidate substance (namely at the presence of nuclear transport-inducing substance 6 only). Accordingly, the substance reducing the signal of reporter protein 3 can be estimated as the nuclear transport-inhibiting substance.

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For introducing Probe I (11) into the cytosol (C) and localizing Probe II (12) in the nucleus (N) in this screening method too, a method for introducing the polynucleotides expressing pair of probes 1 into cell may be applied. Alternatively, by introducing the polynucleotides expressing pair of probes 1 into a non-human animal multipotent cell and the cell is subjected to ontogenesis, Probe I (11) is introduced into the cytosol and Probe II (12) is localized in the nucleus (N) in all cells of this animal or its progeny.

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Embodiments of this invention are described in more detail below by referring to Examples along the attached drawings. Of course, this invention is not limited by the following Examples, and for the details,

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various embodiments are, needless to say, possible.

### Examples

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[Procedures]

#### (1) Construction of Plasmids

The cDNA-encoding N-terminal domain of Rluc (Rluc-N; 1~229 aa) was modified by PCR to introduce the peptide (KFAEYC: SEQ ID NO: 4) to the C terminus of Rluc-N and to introduce the FLAG epitope (DYKDDDDK: SEQ ID NO: 5) to the N terminus of Rluc-N. The cDNA encoding the modified Rluc-N was fused to a cDNA encoding the N-terminal splicing domain of DnaE (DnaE-N; 1~123 aa) with a native *Hind*III site. The cDNA of C terminus of DnaE-N was fused to a cDNA of a nuclear localization signal [NLS; (DPKKKRKV)<sub>3</sub>: SEQ ID NO: 1] with the *Nco*I site.

The cDNA-encoding C-terminal domain of Rluc (Rluc-C; 230~311 aa) was modified by PCR to introduce the peptide, FNLSH (SEQ ID NO: 6), and the unique enzyme site, *Mun*I, to the N terminus of Rluc-C and to introduce a linker (GGGGSG: SEQ ID NO: 7) and a unique enzyme site, *Not*I, to the C terminus of Rluc-C.

The cDNA encoding AR (1~918 aa) was modified by PCR to add a unique enzyme site, *Not*I, at its N-terminal end and *Xho*I at its C-terminal end. The cDNA encoding the modified Rluc-C was fused to a cDNA encoding the C-terminal splicing domain of DnaE (DnaE-C; 1~36 aa) with the *Mun*I site and to cDNA encoding the full AR with *Not*I.

The cDNAs were subcloned into the expression vector pcDNA 3.1(+) (Invitrogen) at the unique enzyme sites *Bam*HI and *Xho*I.

The PCR products were sequenced to ensure fidelity with a BigDye Terminator Cycle Sequencing kit and a genetic analyzer ABI Prism310 (PE Biosystems).

## (2) Cell Culture and Transfection

COS-7 cells were cultured in DMEM supplemented with 10% steroid-free FBS (a charcoal-extracted FBS) and 1% penicillin/streptomycin at 37°C in 5% CO<sub>2</sub>.

The cells seeded in 12-well culture plates were transfected with 2 µg of constructed plasmids using a lipofection reagent, lipofectAMINE2000 (Invitrogen).

## (3) Western Blot Analysis

COS-7 cells were transfected with either pcRDn-NLS or pcDRc-AR and incubated for 24 h. The cells were washed once in PBS and lysed in 200 µl of lysis buffer (1% SDS/10% glycerol/10% 2-mercaptoethanol/0.001% bromophenol blue/50 mM Tris-HCl, pH 6.8). Equal amounts of the samples were electrophoresed in 6% acrylamide gels, transferred to nitrocellulose membrane, and blotted with mouse anti-AR antibody (Santa Cruz Biotechnology). The blots were incubated with alkaline phosphatase-conjugated secondary antibodies and visualized by chemiluminescence (New England Biolabs).

## (4) Immunocytochemistry

COS-7 cells were cultured on microscope glass slides (2 × 10<sup>5</sup> cells per slide) and were transfected with the constructed plasmids. The transfected cells were fixed with a 3% paraformaldehyde solution. The cells were blocked with 0.2% fish skin gelatin and then incubated with mouse anti-AR antibody (Santa Cruz Biotechnology) or mouse anti-FLAG antibody (Sigma). The antibodies were reacted with Cy-5-conjugated secondary antibody, and then recorded by using a confocal laser-scanning microscope (LSM510; Zeiss) fitted with 647 nm cut-off filter and an 665 nm I.P. filter.

#### (5) Cell-Based in Vitro Assay

COS-7 cells were cotransfected with the plasmids and incubated for 12 h. The medium was replaced with DMEM supplemented with 10% FBS, and 24 h after the replacement, steroid hormones or synthetic chemicals were added to each well.

After the COS-7 cells were extensively incubated for 2 hours, the cells were harvested, and luciferase activity was evaluated by using the *Renilla* luciferase assay kit (Promega) with a luminometer (Minilumat LB9506; Berthold, GmbH) with an integration time of 20 sec.

#### (6) In Vivo Imaging of Living Mice

The COS-7 cells were transfected with the constructed plasmid pcRDn-NLS and pcDRc-AR, respectively, or cotransfected with pcRDn-NLS and pcDRc-AR. The cells were harvested after incubation in the DMEM with 10% FBS for 12 h after transfection. The cells were suspended in DMEM, and an aliquot of  $1 \times 10^6$  cells was implanted in four different sites on the back of anesthetized BALB/c nude mice (female, 5 weeks old, about 17 g body weight).

Twelve hours after cell implantation, 100  $\mu$ l of DHT (100  $\mu$ g/kg of body weight) or 1.0% (vol/vol) DMSO (vehicle) was injected i.p. Two hours after injection, 100  $\mu$ l of coelenterazine (2.8 mg/kg of body weight) was injected i.p., and the mice were imaged at 2-min intervals.

For examining DHT dependence on the Rluc activity, two groups of nude mice were injected with the COS-7 cells ( $1 \times 10^6$  cells) cotransfected with plasmids pcRDn-NLS and pcDRc-AR directly into the backs of the mice. Of the two groups, the first mouse group was injected i.p. with 100  $\mu$ l of 1.0% (vol/vol) DMSO (vehicle). The second group was injected with 100  $\mu$ l of DHT (100  $\mu$ g/kg of body weight). After 3 h, 100  $\mu$ l of coelenterazine (1.4 mg/kg of body weight) was injected i.p., and the mice



were imaged 10 min later ( $n = 4$ ).

For testing the brains of mice, COS-7 cells ( $1 \times 10^5$  cells) cotransfected with plasmids pcRDn-NLS and pcDRc-AR were implanted in the forebrain of the nude mice at a depth of 3 mm through a 1-mm burrhole. Soon after implantation, the first and second mouse groups were injected i.p. with 100  $\mu$ l of 1.0% DMSO. The third and fourth mouse groups were injected i.p. with 100  $\mu$ l of procymidone and polychlorinated biphenyls (PCB) (10 mg/kg of body weight) dissolved in 1.0% DMSO, respectively.

One hour after injection, the second and third groups of mice were injected i.p. with 100  $\mu$ l of DHT (10  $\mu$ g/kg of body weight) in 1.0% DMSO. Two hours after injection, 10  $\mu$ l of coelenterazine (0.14 mg/kg of body weight) was injected intracerebrally in the mice, and representatives from each mouse group ( $n = 3$ ) were imaged in 2-min intervals.

All mice were imaged by using a cooled CCD camera (IVIS100 system, Xenogen). Photons emitted from the cells implanted in the mice were collected and integrated for a period of 1 min. Images were obtained by using LIVING IMAGE software (Xenogen). To quantify the measured light, regions of interest were drawn over the cell-implanted area, and the mean luminescence intensities (photons per sec per  $\text{cm}^2$ ) were evaluated.

#### <Example 1>

To ensure that AR connected to the C-terminal domains of RLuc and DnaE (namely, Probe I) is correctly localized in intracellular organelles within animal cells, the COS-7 cells were transiently transfected with pcDRc-AR expression vector.

In the absence of DHT, Probe I was predominantly in cytoplasmic (Fig. 2a-1), whereas addition of DHT resulted in the nuclear localization of Probe I (Fig. 2a-2).

In contrast, Probe II, the N-terminal domains RLuc and DnaE with



nuclear localization signal (NLS), was permanently localized in the nucleus both in the presence and absence of DHT.

These observations were consistent with the results of Western blot analysis (Fig. 3). In crude extracts of the cells containing Probe I plasmid and Probe II plasmid in the absence of DHT, the AR antibody recognized only a specific component of an unspliced precursor, 115 kDa of AR tagged with Rluc-C and DnaE-C.

In the presence of DHT, however, the AR antibody recognized an unspliced precursor and 139 kDa and 160 kDa of a polypeptides, of which electrophoretic mobility was consistent with the predicted size of the products after protein splicing and splicing intermediate.

Given all these results, it was concluded that protein splicing occurred and the Rluc was reconstituted when the DHT-bound AR was translocated into the nucleus.

#### <Example 2>

Next, to show that the pair of probes of this invention works for quantitative analysis of the extent of AR translocation into the nucleus, we tested the DHT-induced translocation of Probe I with the *in vitro* cell-based assay.

The COS-7 cells were transfected with both pcRDn-NLS and pcDRc-AR or only with pcDRc-AR, and differing concentrations of DHT were added to each microtiter well. The cells were harvested, and their lysates were mixed with a coelenterazine solution.

The luminescence signals increased with increasing the concentration of DHT and were strong enough to discriminate them from background luminescence (Fig. 4).

The results indicate that the pair of probes can be used for quantitative analysis of the extent of AR translocation into the nucleus.

## &lt;Example 3&gt;

The AR translocation into the nucleus with several endogenous hormones and synthetic chemicals was confirmed using the pair of probes of this invention.

5 As shown in Fig. 5, endogenous androgens, such as testosterone and 19-nortestosterone, were found to induce high luminescence intensities over their concentration range from  $10^{-8}$  to  $10^{-5}$  M. The relative intensities with testosterone and 19-nortestosterone with their  $10^{-6}$  M concentration were 72% and 64%, respectively, of those with the same  
10 concentration of DHT (Fig. 5a). Other endogenous steroid hormones,  $17\beta$ -estradiol and progesterone, a synthetic steroid hormone; cyproterone acetate (CPA); and anti-androgens, vinclozolin and flutamide, also gave slight increases in the observed luminescence intensities (Fig. 5b). In contrast, procymidone did not induce any increase in its luminescence  
15 intensity over the concentration range tested.

Procymidone was also found to inhibit the DHT-induced strong luminescence. This indicates that procymidone did not trigger the nuclear import of AR even though it was bound to AR.

20 Of other tested chemicals, a PCB congener (Aroclor 1254) was found to hinder nuclear translocation of AR induced with DHT, whereas o,p'-DDT directed a small quantity of AR to the nucleus.

## &lt;Example 4&gt;

25 Next, it was confirmed that the distribution of chemical compounds in the organs of living animal could be observed using the pair of probes of this invention.

An optical bioluminescence imaging technique using a cooled CCD camera has advantages such as easiness of operation, short acquisition time and simultaneous measurement of plural mice, enabling high-speed  
30 imaging (non-Patent Documents 3 and 21 to 23).

To ensure that luminescence intensities provided by RLuc reconstituted in living mice are strong enough to detect with a CCD camera, 1,000,000 untransfected COS-7 cells (Reference Example), cells transiently transfected with pcRDn-NLS or pcDRc-AR or cells cotransfected with both  
5 pcRDn-NLS and pcDRc-AR were implanted into the coria on each site of the backs of living mice.

After 8-35 minutes, an observed cooled CCD image of mice showed a significant increase in the luminescence signal only from the site implanted with the cells cotransfected with pcRDn-NLS and pcDRc-AR (Figs.  
10 6-8). The luminescence intensity obtained from the cotransfected cells reached the maximum at 15 min, which was 20 times higher than the background luminescence intensity from the cells transfected with either pcRDn-NLS or pcDRc-AR alone. The results demonstrate that high levels of bioluminescence can be detected from the implanted cells in living mice  
15 upon reconstitution of split RLuc.

#### <Example 5>

To show the DHT-dependent nuclear import of AR in living mice, COS-7 cells transiently cotransfected with pcRDn-NLS and pcDRc-AR were  
20 implanted on the back of mice.

One group of mice (n=4) were stimulated with 1.0% DMSO (vehicle) for 2 hours, whereas the other group of mice (n=4) were stimulated with DHT (100 µg/kg of body weight) for the same period. Luminescence intensities obtained from the mice with DHT stimulation were 3.5 times  
25 higher than that with vehicle stimulation (Figs. 9 and 10).

The results demonstrate that it is possible to image and quantitatively evaluate the difference in the extent of AR translocation in living mice in the presence of DHT relative to its absence.

#### 30 <Example 6>

Further, the effects of inhibitors on AR translocation into the nucleus in the mouse brain were examined using the pair of probes of this invention.

5 The cells cotransfected with pcRDn-NLS and pcDRc-AR were implanted in the mouse brain at a depth of 3 mm, and i.p. injection was performed with  $10^{-7}$  M DHT alone or different hormone mixtures of further adding procymidone or PCB at various concentrations to 1.0% DMSO.

10 Induction of AR nuclear import by DHT resulted in a significant increase in photon count from the brains as compared to that with vehicle (Figs 11, 12).

Average from three mice indicates that the amount of the increase in bioluminescence was large in comparison to the luminescence intensity of the control. Stimulation with DHT together with procymidone and PCB, respectively, resulted in a decrease in luminescence intensity, 15 demonstrating that procymidone and PCB had an ability to pass through the blood-brain barrier within 2 hours and hindered nuclear import of AR.

#### <Example 7>

20 Translocation of glucocorticoid receptor (GR) into the nucleus was examined using a pair of probes of this invention.

Plasmid pcDRc-AR in Examples 1 to 6 was cleaved with restriction endonucleases NotI and XhoI to remove the cDNA encoding AR, and a cDNA encoding glucocorticoid receptor (GR) was inserted at NotI and XhoI sites (Fig. 13). The same experiment as in case of AR was performed using 25 the thus-constructed plasmid.

The cDNA-encoding N-terminal domain of Rluc (Rluc-N; 1-229 aa) was modified by PCR to introduce the peptide (KFAEY: SEQ ID NO: 8) to the C terminus of Rluc-N, and the cDNA-encoding C-terminal domain of Rluc (Rluc-C; 230-311 aa) was modified by PCR to introduce the peptide 30 (CFNLSH: SEQ ID NO: 9) to the N terminus of Rluc-C.

(1) Confirmation of GR localization

For confirming that GR connected to the C-terminal domains of RLuc and DnaE is correctly localized in intracellular organelles within  
5 animal cells, NIH3T3 cells were transfected with pcDRc-GR expression vector.

In the absence of corticosterone, Probe I was predominantly in cytoplasmic (Fig. 14, upper column, "Corti-"), whereas addition of corticosterone resulted in the nuclear localization of Probe I. (Fig. 14,  
10 second column, "Corti+").

(2) Quantification of GR translocation into the nucleus

Next, to show that the pair of probes of this invention works for quantitative analysis of the extent of GR translocation into the nucleus, we  
15 tested the corticosterone -induced translocation of Probe I with the *in vitro* cell-based assay.

NIH3T3 cells were cotransfected with pcRDn-NLS and pcDRc-GR, and different concentrations of corticosterone were added to each microtiter well. The cells were harvested, and their lysates were mixed with a  
20 coelenterazine solution.

The luminescence signals increased with increasing the concentration of corticosterone and were strong enough to discriminate them from background luminescence.

The GR translocation into the nucleus with several synthetic  
25 chemicals was confirmed using the pair of probes of this invention. As shown in Fig. 15, dexamethasone, progesterone and cortisol were found to induce the GR translocation into the nucleus.

(3) GR translocation into the nucleus in living mice

30 Next, it was verified that GR translocation into the nucleus within

living mice could be observed using the pair of probes of this invention. NIH3T3 cells cotransfected with PcRDn-NLS and pcDRc-GR were implanted into the coria on each sites of the backs of living mice (Fig. 16). Twelve hours later, the mice were forced to swim in a water bath for 10 minutes to exert stress thereon. One hour later, coelenterazine was administered, and the luminescence intensities were measured with a CCD camera.

As shown in Fig. 16, the significant increase in luminescence signal could be observed from the stressed-mouse ("swimming (+)" in Fig. 16) relative to the unstressed-mouse ("swimming (-)" in Fig. 16).

10 The results demonstrate that the physiological increase in corticosterone concentration of the living mice by stress stimulation can be detected with high accuracy upon reconstitution of RLuc.

#### <Example 8>

15 Translocation of Sterol Regulatory Element-Binding Protein-2 (SREBP-2) into the nucleus was examined using a pair of probes of this invention in the same manner as in Example 7.

Plasmid pcDRc-AR in Examples 1 to 6 was cleaved with restriction endonucleases NotI and XhoI to remove the cDNA encoding AR, and a cDNA encoding SREBP-2 was inserted at NotI and XhoI sites thereby constructing pcDRc-SREBP (Fig. 17).

The cDNA-encoding N-terminal domain of RLuc (Rluc-N; 1-229 aa) was modified by PCR to introduce the peptide (KFAEYC: SEQ ID NO: 4) to the C terminus of Rluc-N, and the cDNA-encoding C-terminal domain of RLuc (Rluc-C; 230-311 aa) was modified by PCR to introduce the peptide (CFNLSH: SEQ ID NO: 9) to the N terminus of Rluc-C.

#### (1) Confirmation of SREBP-2 localization

For confirming that SREBP-2 connected to the C-terminal domains of RLuc and DnaE is correctly localized in intracellular organelles in



animal cells, COS-7 cells were transfected with pcDRc-SREBP expression vector.

In the presence of cholesterol (Fig. 18, "+chol"), the probe with SREBP-2 was predominantly in cytoplasmic (Fig. 18(1)). On the other hand, in the absence of cholesterol (Fig. 18, "-chol") resulted in the nuclear localization of the probe. (Fig. 18(2)-(4)).

#### (2) Quantification of SREBP-2 translocation into the nucleus

Translocation of SREBP-2 into the nucleus was qualified in the same manner as in Example 7. COS-7 cells were cotransfected with pcRDn-NLS and pcDRc-SREBP, and different concentrations of cholesterol were added to each microtiter well. The cells were harvested, and their lysates were mixed with a coelenterazine solution.

The luminescence signals increased with decreasing the concentration of cholesterol (Fig. 19) and were strong enough to discriminate them from background luminescence. These results indicate that the extent of SREBP-2 translocation into the nucleus can be detected using the luminescence intensity as an index.

#### <Example 9>

Translocation of Signal Transducer and Activator of Transcription 3 (STAT-3) into the nucleus was examined using a pair of probes of this invention in the same manner as in Examples 7 and 8.

Plasmid pcDRc-AR in Examples 1 to 6 was cleaved with restriction endonucleases NotI and XhoI to remove the cDNA encoding AR, and a cDNA encoding STAT-3 was inserted at NotI and XhoI sites thereby constructing pcDRc- STAT-3 (Fig. 20).

#### (1) Confirmation of STAT-3 localization

For confirming that STAT-3 connected to the C-terminal domains of RLuc and DnaE is correctly localized in intracellular organelles in animal



cells, HEK293 cells were transfected with pcDRc-STAT3 expression vector.

In the absence of oncostatin M (OSM), a ligand, the probe with STAT3 was predominantly in cytoplasmic (Fig. 21, "OSM-"), whereas addition of OSM resulted in the nuclear localization of the probe (Fig. 21, "OSM+").

## (2) Quantification of translocation of STAT-3 into the nucleus

Translocation of STAT-3 into the nucleus was qualified in the same manner as in Examples 7 and 8.

HEK293 cells were cotransfected with pcRDn-NLS and pcDRc-STAT3, and different concentrations of OSM were added to each microtiter well. The cells were harvested, and their lysates were mixed with a coelenterazine solution.

The luminescence signals increased with decreasing the concentration of cOSM (Fig. 21) and were strong enough to discriminate them from background luminescence. These results indicate that the extent of STAT-3 translocation into the nucleus can be detected using the luminescence intensity as an index.

## Industrial Applicability

As has been thus far described in detail, this invention provides a method for non-invasive imaging of protein nuclear transport in living cells or animals. Further, this invention provides a method for quantifying the extent of protein translocation into the nucleus conveniently. The method for detecting and quantifying protein nuclear transport using the pair of probes of this invention is, unlike ordinary fluorescence techniques, free of background signals as a bioluminescence method, and makes it possible to perform the detection with high accuracy and high sensitivity.

Accordingly, its application is expected to therapy, identification of toxics, development of drugs and the like.

The pair of probes for detecting protein nuclear transport in the first invention comprises Probe I in which a protein whose nuclear transport is to be detected or quantified is connected to an N-terminal end or a C-terminal end of a fusion protein [intein-C/reporter protein-C] wherein at least a C-terminal side polypeptide of an intein and a C-terminal side polypeptide of a reporter protein are connected in this order, and Probe II in which a nuclear localization signal is connected to an N-terminal end or a C-terminal end of a fusion protein [reporter protein-N/intein-N] wherein at least the remaining N-terminal side polypeptide of the reporter protein and the remaining N-terminal side polypeptide of the intein are connected in this order.

Likewise, the pair of probes for detecting protein nuclear transport in the second invention comprises Probe I in which a protein whose nuclear transport is to be detected or quantified is connected to an N-terminal end or a C-terminal end of a fusion protein [reporter protein-N/intein-N] wherein at least a N-terminal side polypeptide of a reporter protein and a N-terminal side polypeptide of an intein are connected in this order, and Probe II in which a nuclear localization signal is connected to an N-terminal end or a C-terminal end of a fusion protein [intein-C/reporter protein-C] wherein at least the remaining C-terminal side polypeptide of the intein and the remaining C-terminal side polypeptide of the reporter protein are connected in this order.

When these pairs of probes are introduced into cells, Probe I resides in the cytosol, and Probe II is localized in the nucleus. When the protein in Probe I recognizes and binds to a bioactive substance, Probe I translocates from the cytosol into the nucleus, and approaches Probe II localized in the nucleus. Upon the approach, the divided inteins in Probe I and Probe II are cut out by splicing, thereby the reporter protein is

reconstituted. Therefore, the protein translocation into the nucleus induced by the bioactive substance can be detected with high accuracy with a measurement of the signal of the reporter protein.

5 In the pair of probes for detecting protein nuclear transport in the third invention, the intein is DnaE derived from blue-green algae, so that the intein is surely cut out automatically.

10 In the pair of probes for detecting protein nuclear transport in the fourth invention, the reporter protein is luciferase, a luminous catalytic enzyme. When luciferase is reconstituted upon occurrence of splicing between Probe I and Probe II, the active center is formed to emit light easily be detectable with a luminometer.

15 The method for detecting and quantifying protein nuclear transport in the fifth invention comprises causing Probe I of the pair of probes and the bioactive substance to coexist in the cytosol, localizing Probe II in the nucleus, and measuring a signal of the reporter protein within the nucleus. When the protein recognizes and binds to the bioactive substance, Probe I translocates from the cytosol into the nucleus. Then, Probe I approaches Probe II localized in the nucleus, the divided inteins in Probe I and Probe II are cut out by splicing to reconstitute the reporter protein. Accordingly, 20 by the signal of the reporter protein, the protein translocation into the nucleus induced by the bioactive substance can be detected surely with high accuracy. It is also possible to quantify the protein translocation into the nucleus.

25 In the method for detecting and quantifying protein nuclear transport in the sixth invention, polynucleotides expressing the pair of probes are introduced into a cell, whereby it is possible that Probe I and the bioactive substance are coexisted in the cytosol and Probe II is localized in the nucleus. In the method for detecting and quantifying protein nuclear transport in the seventh invention, polynucleotides 30 expressing the pair of probes are introduced into a non-human animal

multipotent cell and the cell is subjected to ontogenesis, whereby it is possible that Probe I and the bioactive substance are coexisted in the cytosol and Probe II is localized in the nucleus in all cells of this animal or its progeny.

5           The method for screening a nuclear transport -inducing substance in the eighth invention comprises introducing Probe I of the pair of probes into the cytosol, localizing Probe II in the nucleus, introducing a nuclear transport-inducing candidate substance into the cytosol, and measuring a signal of the reporter protein in the nucleus. Binding of the candidate  
10 substance with the protein in Probe I induces the protein translocation into the nucleus, Probe I approaches Probe II localized in the nucleus, and the divided inteins in Probe I and Probe II are cut out by splicing, thereby the reporter protein is reconstituted. Accordingly, by measuring the signal of the reporter protein, it can be judged with high accuracy whether or not  
15 the candidate substance acts on the protein as the nuclear transport -inducing substance.

          The method for screening a nuclear transport-inhibiting substance in the ninth invention comprises introducing Probe I of the pair of probes into the cytosol, localizing Probe II in the nucleus, introducing a nuclear  
20 transport-inhibiting candidate substance into the cytosol, further introducing a nuclear transport-inducing substance into the cytosol, measuring a signal of the reporter protein in the nucleus, and comparing the signal with a signal of the reporter protein obtained by introducing only the protein nuclear transport-inducing substance into the cytosol. In the  
25 case of the candidate substance acting as the nuclear transport -inhibiting substance, the candidate substance first binds to the protein, and hence the nuclear transport-inducing substance cannot bind to the protein. Consequently, the signal of the reporter protein is reduced in comparison to the signal of the reporter protein in introducing the nuclear  
30 transport-inducing substance only into the cytosol. On the other hand,

where the candidate substance does not act as the nuclear transport-inhibiting substance, the candidate substance does not bind to the protein, and therefore the nuclear transport-inducing substance in the cytosol binds to the protein, which leads translocation of the protein into the nucleus. As a result, the signal of the reporter protein becomes equal when only the nuclear transport-inducing substance is introduced into the cytosol.

In the screening method in the tenth invention, polynucleotides expressing the pair of probes are introduced into the cell for introducing Probe I into the cytosol and localizing Probe II in the nucleus. In the screening method in the eleventh invention, polynucleotides expressing the pair of probes are introduced into a non-human animal multipotent cell and the cell is subjected to ontogenesis, whereby it is possible that the Probe I is introduced in the cytosol and Probe II is localized in the nucleus in all cells of this animal or its progeny.

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